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Production of 1,2-propanediol from complex biomass

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"Ég lýsi því yfir að ég ein er höfundur þessa verkefnis og að það er afrakstur eigin rannsókna".

____

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Það staðfestist að verkefni þetta fullnægir að mínum dómi kröfum til prófs í námskeiðum LOK1126 og LOK1226.

____

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Abstract

1,2-propanediol (1,2-PD) is an organic compound with many applications, ranging from food additives to the manufacture of cosmetics and plastics. Currently, 1,2-PD is industrially produced by the hydration of propylene oxide, a non-renewable petrochemical derivative, but this is not the only method of obtaining 1,2-PD. Using a variety of microbial strains it is possible to produce 1,2-PD by converting de-oxy sugars to yield the S enantiomer or the fermentation of hexoses and pentoses to yield the R entantiomer. With this methods, there is the potential to use a more environmentally friendly production process by utilizing the sugars found in renewable biomasses for agriculture. *Thermoanaerobacterium thermosaccarolyticum HG-8* was used to produce 1,2-PD from glucose and several lignocellulosic biomasses to compare the end product yields of different starting materials and their maximum potential yield. Aside from 1,2-PD, the end products produced were ethanol, acetate, lactate, and H_2. Of the hydrolysates tested, only glucose, whatman paper, Timothy grass and potato leaf produced end product above control levels. The average production yield of 1,2-PD was 4,25 mM, with the highest production occurring with glucose (20 mM), which is known to produce high yields. Of the biomasses tested, whatman paper (5g dry weight/L) produced the highest yield of 1,2-PD. Kinetic experiments using glucose and Timothy grass determined that the maximum potential yield of 1,2-PD occurred after 48 and 21 hours, respectively.

Key words: Biofuel – 1,2-propanediol – complex biomass – HG-8 - Biotechnology

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Útdráttur

1,2-própandíol (1,2-PD) er lífrænt efnasamband með mörg notkunargildi, frá aukaefnum í mat yfir í framleiðslu á snyrtivörum og plasti. Eins og er, er 1,2-PD framleitt með vötnunprópýlenoxíðs, sem er óendurnýjanlegt jarðefna (petrochemical) afleiða sem unnin er úr jarðolíu. Þetta er þó ekki eina leiðin til þess að framleiða 1,2-PD. Með því að nota mismunandi örverustofna er möguleiki á að framleiða 1,2-PD með því að breyta de-oxý sykrum. Með þessari aðferð er hægt að nota umhverfisvænni framleiðsluferli með því að nýta sykrurnar sem finnast í endurnýtanlegum lífmassa frá landbúnaði. *Thermoanaerobacterium thermosaccarolyticum HG-8* var notuð til þess að framleiða 1,2-PD. De-oxý sykru (glúkósi) og nokkrum gerðum af lífmassa var borið saman til þess að sjá muninn á loka afurð frá mismunandi byrjunar efni og þeirra hæstu uppskeru. Fyrir utan 1,2-PD, voru framleiddar aðrar loka afurðir, það voru etanól, asetat, laktat og H_2. Af þeim vatnsrofum sem prófuð voru, var aðeins glúkósí, Whatman pappír, Timothy gras og kartöflulauf sem framleiddu loka afurðir. Meðal uppskera fyrir 1,2-PD var 4,25 mM, þar sem að mesta framleiðslan átti sér stað með glúkósa (20 mM), sem er þekktur fyrir háa uppskeru á 1,2-PD. Af lífmassa sem var prófaður, var whatman paper með hæstu uppskeruna af 1,2-PD. Kinetic tilraunirnar með glúkósa og Timothy grass ákvörðuðu það hvenær mesta uppskeran átti sér stað. Mesta uppskeran átti sér stað eftir 48 og 21 klukkustundir.

Lykilorð: Lífeldsneyti – 1,2-própandíol – Flókinn lífmassi – HG-8 - Líftækni

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1 Research Objectives

The objective of this thesis is to build upon previous research that determined that producing 1,2-PD in a greener and more cost effective way through the use of agricultural biomass as a renewable resources was possible. The research conducted intends to determine if 1,2-PD can be produced from lignocellulosic biomass using *T. thermosaccharolyticum HG-8*, compare different sources of biomass with reference to the production yield, and to investigate the effects of the liquid to gas ratio on production.

The following research questions were purposed:

- Can 1,2-PD be produced with HG-8 using lignocellulosic

  biomass?

- Can agriculture waste be useful to produce biofuels, such as

  1,2-PD?

2 Introduction

2.1 1,2-propanediol

1,2-propanediol (1,2-PD), also known as α-propylene glycol, and propane-1,2-diol, is a three-carbon diol with a stereogenic center at the central carbon atom existing as two enantiomers: R(-)-1,2-propanediol and S(-)-1,2-propanediol (Figure 2). It is a diol with a high boiling point.

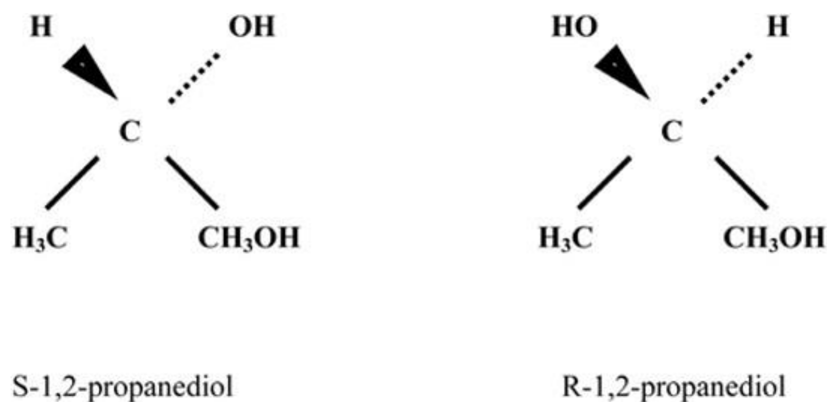

Figure 1 - Chemical structure of 1,2-PD (Saxena, Anand, Saran, Isar, & Agarwal, 2010a)

1,2-PD is an important chemical with multiple applications. It can be used as an anti- freeze agent, for chemical application, and within consumer products and foods. As an important chiral synthon, 1,2-PD is used in the food industry as a natural food additive and in the pharmaceutical industry to manufacture chiral pharmaceutical products. However, the use of R and S-1,2-PD is limited as they are expensive (Cameron & Cooney, 1986; Chen, Zidwick, & Rogers, 2013; Saxena, et al., 2010b). (Cameron, Altaras, Hoffman, & Shaw, 1998)

Currently, 1,2-PD is industrially produced by the hydration of propylene oxide (Xiao et al., 2015), which is a non-renewable petrochemical derivative (Altaras, Etzel, & Cameron, 2001; Metzger, 2009). 1,2-PD can, however, be produced from renewable resources using microbes, such as *Thermoanaerobacterium thermosaccharolyticum*. Other organisms that can produce 1,2-PD can be seen in Table 1.

Table 1 – Features of selected 1,2-PD producing organism

Organism	Substrate (and conc if available)	Yield (1,2-PD)	Reference
Bacteroides ruminocola	Xylose, arabinose, rhamnose		(Turner & Roberton, 1979)
Caldicelluliruptor bescii	L-rhamnose	0,73 mM	(Ingvadottir, Scully, & Orlygsson, 2017)
Caldicelluliruptor owensensis	L-rhamnose	7,66 mM	(Ingvadottir, Scully, & Orlygsson, 2017)
Clostridium sphenoides	Rhamnose, fucose, glucose, fructose, cellobiose, mannose	Rhamnose: 72,6 mM Fucose: 68,6 mM	(Turner & Roberton, 1979)
Escherichia coli	Rhamnose, fucose, glucose	0,25 g from 10 g/l of glucose	(Turner & Roberton, 1979)
Lactobacillus brevis	Glycerol	0,5 M acetic acid, 0,5 M 1,2-PD	(Obradors, Badía, Baldomà, & Aguilar, 1988)
Lactobacillus buchneri	Glycerol	0,5 M acetic acid, 0,5 M 1,2-PD	(Obradors et al., 1988)
Saccharomyces cerevisae	Glucose	0,24 g/l	(Turner & Roberton, 1979)
Salmonella typhimurium	Rhamnose, fucose, methyl pentose	1 M of 1,2-PD/M of sugar into the medium	(Obradors et al., 1988)
Thermoanaerobacterium thermosaccharolyticum	Glucose, xylose, mannose, cellobiose, fructose, galactose, arabinose, lactose	Glucose: 9,0 g/l Galactose: 3,5 g/l	(Cameron D.C. et al., 1998)

Several strains of *T. themosaccharolyticum* can produce *R*(-)-1,2-PD from a variety of sugars, such as D-glucose and D-xylose (Chen et al., 2013; Saxena et al., 2010b). These sugars are two of three major sugars that can be obtained from cellulosic biomass; the third sugar being L-arabinose. Cellulosic biomass can, therefore, be used to produce 1,2-PD using sugars derived from the processing of low-cost biomass (Chen et al., 2013; Saxena et al., 2010b).

The pathway to (*R*)-1,2-PD with *T. thermosaccharolyticum* begins with dihydroxyacetone phosphate (DHAP), which is dephosphorylated to methylglyoxal. The methylglyoxal is then reduced to hydroxyl acetone with a fraction being converted to D-lactate (Sánchez-Riera, Cameron, & Cooney, 1987). The hydroxyl acetone is further reduced to (*R*)-1,2-PD (Figure 2) (Cameron et al., 1998). Many other microorganisms, such as bacteria and yeast, produce *S*(+)-1,2-PD from fairly uncommon sugars, including L-rhamnose and L-fucose (Douglas C. Cameron & Cooney, 1986). However if the production of 1,2-PD is done by chemical means, the end product will be a mixture of *R* and *S* isomers (Chen et al., 2013; Saxena et al., 2010b).

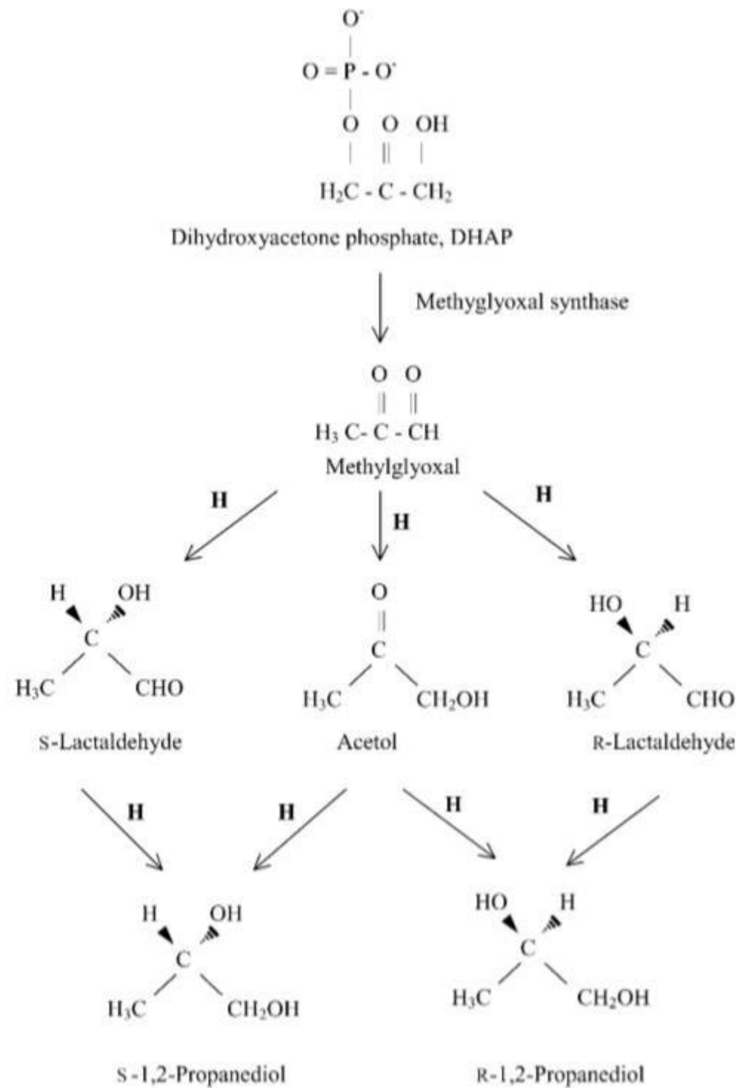

Figure 2 - The pathways to (R)- and (S) - 1,2-PD (Bennett & San, 2001)

The optimal production of 1,2-PD occurs under specific conditions, taking into account the temperature, pH, gas phase composition and substrate. The optimal conditions to produce 1,2-PD using *T. thermosaccharolyticum HG-8* is at 60°C with a pH of 6 and the use of N_2 and yeast extract. It has been shown that yeast extract has an important effect on the

production. The temperature has an important role in the production, as well, because at higher than optimal temperatures, the production of (?) lactate is decreased and 1,2-PD increased (Chen et al., 2013).

In the early experiments of fermentation of 1,2-PD, *T. thermosaccharolyticum* did not grow on lactose and D-galactose. Therefore, it was selected to grow on these sugars by multiple serial transfers. 1,2-PD was not detected in these sugars after the adaption of the organism but was more promising in sugars such as arabinose and xylose. There the production of 1,2-PD had almost the same yields in arabinose as in glucose. Using xylose produced half of the yields glucose produced. That was before the optimal conditions were known (Chen et al., 2013; Saxena et al., 2010b). The improvements kept on going and another approach was to use metabolic engineering. Because many tools are available for genetic modification, *Escherichia coli* was chosen as a host organism. The experiments led to the conclusion that *E. coli* could not produce 1,2-PD from glucose, but was able to produce the intermediate methylglyoxal. (Chen et al., 2013).

2.2 Thermophilic anaerobes

Thermophilic anaerobes are bacteria and Archaea that do not require oxygen for growth and grow optimally at high temperatures. These microorganisms are often found in environments that are considered inhospitable to "normal" mesophilic life, including hot springs, volcanic solfataras, as well as artificially warm environments, such as coal refuse piles, compost heaps and nuclear power plant effluent channels. The ability to thrive at elevated temperature is why thermophilic anaerobes are designated extremophiles. They have also been isolated from mesobiotic and psychrobiotic environments, that are at temperatures below 12°C (Wagner & Wiegel, 2008).

2.2.1 Key relationships to environmental conditions

Thermophilic anaerobes can be described with different environmental conditions; temperature, oxygen, and pH. The following chapters describe the conditions that are optimal, minimum, and maximum for these types of organisms.

2.2.1.1 Temperature relations

Thermophilic anaerobes can be categorized according to their optimal and maximum growth temperatures (Table 2). Although there are bacteria and archaea that grow optimally at moderate temperatures, they have a maximal growth temperature at 50°C, and are described as thermotolerant. The highest temperature a growing hyperthermophile has been isolated from was 122°C under increased pressure. This was the *Methanopyrus Kandleri* strain (Wagner & Wiegel, 2008).

Table 2 - Categories for temperature relations (Doi, 2008; Wagner & Wiegel, 2008)

Extremophile	**Optimal growth temperature** (T_{opt}, °C)
Mesophile	10°C-45°C
Thermotolerant	Maximum at 50°C
Moderate Thermophile	50°C-64°C
Extreme Thermophile	65°C-79°C
Hyper Thermophile	≥ 80°C

2.2.1.2 Oxygen relations

As mentioned before, thermophilic anaerobes do not require oxygen to grow or survive. Therefore, many of the environments where thermophilic anaerobes are found are low in oxygen or anaerobic. Although thermophilic anaerobes in general do not require oxygen, some of them, known as facultative aerobes, can live in environments with oxygen.

Anaerobes are unable to use O_2 as an electron acceptor, although they can grow in the presence of it. On the other hand, facultative aerobes are able to use O_2 as an electron acceptor and can survive being exposed to oxygenic atmospheres, that is, if they are metabolically inactive.

These thermophilic anaerobes and facultative aerobic thermophiles can use a variety of compounds as electron acceptors, including CO_2, CO, NO_3^-, NO_2^-, NO, N_2O, SO_4^{-2}, SO_3^{-2}, $S_2O_3^{-2}$, S^0, Fe(III), Mn(IV), and Mo(VI) (Wagner & Wiegel, 2008).

2.2.1.3 pH relations

Thermophilic anaerobes can be categorized by the optimum (pH_{opt}), minimum (pH_{min}) and maximum (pH_{max}) pH level (Table 2).

Table 3 - Categories for pH relations

	Optimum (pH_{opt})	**Minimum (pH_{min})**	**Maximum (pH_{max})**
Acidophilic	$< 5,0$		
Acidotolerant	Circumneutral	$< 4,0$	
Neutrophilic	7,0		
Alkalitolerant	$< 8,5$		$> 8,5$
Alkaliphilic	≥ 8.5		

No known thermophilic anaerobe grows below pH 3,0, but the majority of known thermophilic anaerobes are neutrophilic. Although there are some thermophilic anaerobes with a wide range of pH; *Thermococcus hydrothermalis* for example, which has a growth range of pH 3,5-9,5. Also, there have been some thermophilic anaerobes discovered with a growth range that is less than one pH, namely *Pelotomaculum thermopropionicium*, which has a pH range of 6,7-7,5 (Wagner & Wiegel, 2008).

2.2.2 Thermoanaerobacterium thermosaccharolyticum HG-8

Thermoanaerobacterium thermosaccharolyticum or originally called *Clostridum Thermosaccharolyticum* strain HG-8 is a thermophilic bacterium that is known to ferment sugars to ethanol, lactate, acetate, butyrate, CO_2, H_2 and 1,2-propanediol (Douglas C. Cameron & Cooney, 1986). HG-8 is moderately thermophilic, therefore it grows in temperature 50°C-64°C, but the optimum temperature for this strain is 55°C-60°C. The optimum pH range has not been reported. *T. thermosaccharolyticum* O_2 relationship is anaerobic and the metabolism is chemoorganoheterothroph (Wagner & Wiegel, 2008). The strain has a broad substrate spectrum and can ferment glucose, xylose, arabinose and galactose

to 1,2-propanediol. Although the optimal substrate spectra for production of 1,2-propanediol is glucose (Saxena et al., 2010b).

2.2.3 Clostridum thermoislandicum AK1

Clostridum "thermoislandicum" strain AK1 is a moderate thermophile that was isolated from a hot spring in Grensdalur in South-West Iceland. AK1 falls within Cluster IV of the genus *Clostridium,* although likely represents a novel genus. It has 92,3% similarity with *Ruminococcus champanellensis* within Class *Clostridia.* The strain is a strictly anaerobic, gram-positive, rod-shaped spore former. AK1 is moderately thermophilic, growing in temperatures from 30°C to 60°C with the optimum temperature being 45°C, at which the generation time is 1,9 hours. It also has a broad tolerance to pH, ranging from pH 4,5 to 7,5 with the optimum pH is at 6,5, at which the generation time is 1,6 hours. The strain has a broad substrate spectrum and can ferment hexoses, pentoses, disaccharides, starc,h and pectin. Although it only produces 1,2-propanediol from rhamnose (Orlygsson, 2012; Scully, Ingvadóttir, & Orlygsson, 2015).

2.3 Lignocellulosic biomass

Composed of cellulose, hemicellulose, lignin, and small fraction of extractive acid, salts, and minerals, lignocellulosic biomass is classified as a second generation biomass (Ahring, Licht, Schmidt, Sommer, & Thomsen, 1999). A second generation biomass is mostly waste products from agricultural activities (Jessen & Orlygsson, 2012) and is, therefore, a product that is available in large quantities at low cost (Wright & J.D., 1987).

Lignocellulosic biomass usually contains around 55-75% carbohydrates by dry weight. One of the carbohydrate components, cellulose, is a polymer of glucose. Cellulose has a specific structure, that favors the ordering of the polymer chains into tightly packed, highly crystalline structures. This makes them water insoluble and resistant to depolymerization. Hemicellulose, however, is a branched polymer of glucose or xylose, that is substituted with arabinose, xylose, galactose, fucose, mannose, glucose or glucuronic acid. Therefore, cellulose microfibrils and hemicellulose hydrogen-bonds provide a structural backbone to plant cell walls. These two carbohydrates are potential sources of fermentable sugars (Mosier et al., 2005).

Although biofuel can be produced from lignocellulosic biomass, there is a pretreatment that the biomass needs to go through before it can produce biofuel. The digestibility of the cellulose that is present in the biomass is hindered by many physicochemical, structural and compositional factors and composition factors. For example, lignin which is complex polymer of phenylpropanoid and lignin in the cell wall impedes enzymatic hydrolysis of the carbohydrates he pretreatment uses various techniques to expose the plant fibers and to make it more accessible to the enzyme or acid that converts the carbohydrate polymers into fermentable sugars (Mosier et al., 2005). The goal is to remove lignin and hemicellulose, and reduce the crystallinity of cellulose, to increase the porosity of the lignocellulosic materials (Mosier et al., 2005; Kumar, Barrett, Delwiche, & Stroeve, 2009; McMillan, 1994). The reason why the pretreatment is so important in this process is that, after it has removed all the lignin nd hemicellulose, enzymatic digestibility is increased. Therefore the enzyme can access the biomass macrostructure (Zhao, Cheng, & Liu, 2009). The goal of pretreatment in biomass to biofuel is shown in Figure 3.

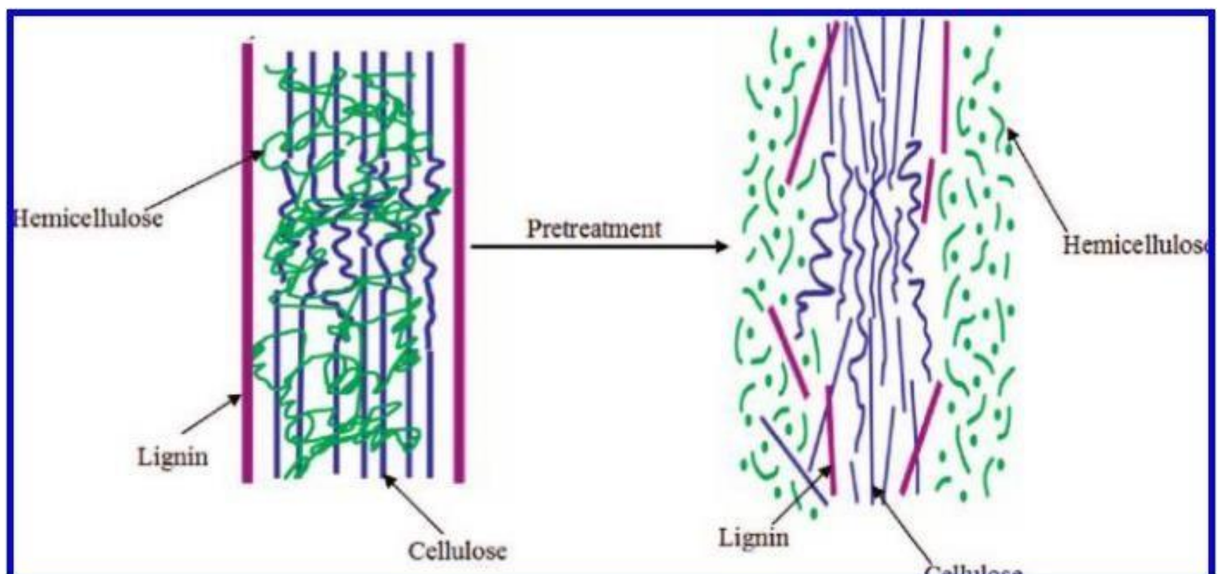

Figure 3 - Schematic of the role of pretreatment in the conversion of biomass to fuel (Kumar et al., 2009).

The crystalline structure of cellulose is disrupted by breaking it down to its corresponding monomers, making them available to microorganisms. This disruption is called acid hydrolysis and is also applicable for hemicellulose. Pretreatment with acid hydrolysis can result in improvement of enzymatic hydrolysis of lignocellulosic biomasses to release

fermentable sugars. The development of dilute-acid hydrolysis has been successful for pretreatment of lignocellulosic materials. Dilute H_2SO_4 has been of the most interest as it is inexpensive and effective. Dilute H_2SO_4 is mixed with biomass to hydrolyze hemicellulose to xylose and other sugars. It continues to break xylose down to form furfural. This pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis. When the H_2SO_4 is added, hemicellulose is removed, which improves the digestibility of cellulose in the residual solids.

The most important thing about the pretreatment is that it meets some requirements, such as improves the formation of sugars or the ability to subsequently form sugars by hydrolysis, avoids the degradation or loss of carbohydrates, and avoids the formation of byproducts that are inhibitory to the subsequent hydrolysis (Zhao, Cheng, & Liu, 2009).

3 Experiment

3.1 Medium

The composition and the preparation of the Basal Mineral (BM) medium has been described by (Sveinsdottir, Baldursson, & Orlygsson, 2009)(Table 1 in Appendix). The BM medium contains 2,0 (g/L) yeast extract in addition to glucose or another carbon source. The inoculum volume was 2% (v/v) for the strain tested, and all tests were performed in triplicates. The medium was added to serum bottles using the Hungate technique (Hungate, 1969). The medium was then autoclaved for 60 min (121°C) before hydrolysate, trace elements, and vitamins were added separately through 0,22 µm syringe filters.

3.2 Organisms and Cultivation

The strain *Thermoanaerobacterium Thermosaccarolyticum* HG-8 (ATCC 10022) was purchased from the American Type Culture Collection (ATCC). It was cultivated in Basal Mineral (BM) medium prepared according to (Orlygsson & Baldursson, 2007) at pH 7 and a liquid-gas ratio of 1:1 without shaking.

3.3 Effect of culture conditions on 1,2-propanediol

The effect of culture conditions on 1,2-propanediol with HG-8 was investigated by examining the effect of inhibitory compounds and the effect of the liquid-gas ratio. This was performed to control the end-product. Instead of ending up with end-products such as ethanol, acetate, lactate, etc., the end-product should be 1,2-propanediol.

3.3.1 Effect of Liquid-gas phase ratio

Liquid-gas ratio was performed in 58,9 mL serum bottles with three different ratios (0,09, 1,00 and 5,62) in triplicates for each ratio and carbon source. It was then incubated at 65°C for 5 days.

3.4 Biomass Hydrolysates

The hydrolysates (HLs) were prepared from Rhubarb (*Rheum rhabarbarum*), Potato leaves (*latin*), Timothy grass (*Phleum pratense*), Lupine (*Lupinus albus*), Whatman no. 1 filter paper, and newspaper.

The biomass for the hydrolysates, rhubarb, potato leaves, and timothy grass, was collected from different places around Akureyri. These biomasses had to be cleaned and dried at 60°C until it was completely dry. Each biomass was shredded and prepared at a concentration of 25 g/L, based on dry weight. Chemical pretreatment was performed using 0,5% sulfuric acid (H_2SO_4) and then autoclaved (121°C for 60 min). After chemical pretreatment the bottles were cooled down to room temperature and pH adjusted to 4,5 before adding cellulase isolated from *Trichoderma reesei*. The hydrolysates were incubated for 68 h at 47,5°C. When they had been incubated for 68 h, the pH was adjusted with NaOH or HCl to pH 7,0. The hydrolysates were centrifuged and sterilized by filtering through 53 µm, 11 µm, 5,3 µm, and 0,22 µm filters into aseptic bottles

3.4.1 Fermentation of hydrolysates

Fermentation of carbohydrates present in the hydrolysates after chemical and enzymatic pretreatment was performed in 58,9 mL serum bottles at a liquid-gas phase ratio of 1:1. The BM medium and inoculum were supplemented with different hydrolysates. Each bottle included BM medium, C_1 (table 2 in appendix), C_2 (table 3 in appendix), carbon source and inoculation. There were also control samples that did not contain hydrolysates. Their only carbon source was yeast extract. Fermentations were carried out for 5 days.

3.5 Kinetic experiments

Fermentation of carbohydrates present in the hydrolysates after chemical and enzymatic pretreatment was performed in 125 mL serum bottles at a liquid-gas ratio of 1:1. The BM medium and inoculum were supplemented with glucose or timothy grass hydrolysate at a final concentration of 5 g/L. Fermentations were carried out for 96 hours.

3.6 Analytical Methods

Volatile end products such as acetate and ethanol as well as hydrogen were measured using GC-FID or GC-TCD, repetitively, as has been described by (Orlygsson & Baldursson, 2007). 1,2-propanediol was measured colorimetrically using the method in Jones, 1957, with the modifications described in E M Ingvadottir, Scully, & Orlygsson, 2017.

Proximate analysis of the biomass was carried out according to standard methods. Protein concentration in the biomass used for the hydrolysates was measured using the

Kjeldahl method. Water and fat concentration of the biomass was measured using Soxhlet extraction method. Finally, mineral concentration of the biomass was measured using the ash method.

3.6.1 Kjeldahl method

Dried biomass was weighed on paper, using approximately 0,5 g per biomass. This was done in triplicates for each biomass. The samples were all put in test tubes along with 0,15 g of $CuSO_4$. Each test tube was filled up with 12 mL of sulfuric acid and boiled at 420°C with suction for 2 hours. After 2 hours, the samples had to cool down before putting 25 mL of distilled water into each test tube. Each sample was then put into Kjeltec distiller, along with 25 mL of Bromoeresol green, and methyl red acid in an Erlenmeyer flask. Before distillation began, 20 mL of NaOH was added to the test tubes. After distillation each sample was titrated with 0,10000 M hydrochloric acid.

3.6.2 Soxhlet extraction method

Firstly, bowls were prepared by drying them in oven at 104°C for 1 hour and cooled down in a desiccator. Laboratory sand was added to the bowls and in each one, was weighed 5 g of biomass, repeated in triplicates. The sand and sample were mixed together and put in the oven at 104°C for 4 hours. After that the bowls were removed and put in a desiccator to cool down and then weighed. Each sample was removed from the bowls and put in a filter cup with a cotton ball put on top. The filter cup was put in the Soxhlet machine and Florence flasks, that had already been sterilized, were attached to the machine. In each Florence flask was put 150 mL of n-hexane that was distilled away after the filter cups had been in the Soxhlet machine for four hours. Florence flasks were then dried in the oven for 30 minutes at 104°C, allowed to cool, and then weighed.

3.6.3 Ash method

Crucibles had been dried in the oven for one hour and allowed to cool in a desiccator. All crucibles were weighed and 1,0 g of biomass was added to them. All the samples were put in an oven at 104°C for one hour, then burned over a Bunsen burner, and subsequently transferred to an ash oven at 550°C for 4 hours. All crucibles were cooled down in a desiccator for 30 minutes and weighed. All biomasses were done in triplicates.

4 Results

4.1 Proximate Analysis

Results from all the proximate analysis can be seen in Table 4. The analysis was done with lignocellulosic biomasses based on dry material. All the analysis shown in the table were done in triplicates and the average percentage of ash content, Soxhlet and Kjeldahl was used. Standard deviation was calculated for all samples. Percentage of carbohydrates was calculated from the results of percentage of the other analysis.

	Ash	*Soxhlet*	*Kjeldahl*			*Carbohydrates*
Potato leaf	12.53±0.40	2.72±0.14	14.25±0.17			70.52
Lupine	7.10±1.07	0.95±0.06	8.85±0.42			83.09
Rhubarb	11.93±0.10	3.30±0.47	17.97±0.43			66.79
Timothy grass	13,34±1,64	1,89±0,14	2,37±0,51			82.40
Whatman paper	0,05±0,00	0,00	0	0	0	99.95
Newspaper	1,23±0,15	0,00	0	0	0	98.77

Table 4– The table shows results for the average percentage and standard deviation of the three replicates for Ash content, Soxhlet, Kjeldahl, but only average percentage for Carbohydrate based on the biomass hydrolysate.

The ash content calculations represent the percentage of mineral content. Each biomass shows different results for ash content. Timothy grass shows the highest percentage of ash content [13,34 ± 1,64%], while Whatman paper only has 0,05 ± 0% ash content. The percentage average for rhubarb is 11.93%, but the standard deviation is 0,10, indicating that the results were more precise for rhubarb than for Timothy grass.

The Soxhlet calculation represents the percentage of lipids. The results show that rhubarb has the highest lipids of the six biomasses, but also has the highest standard deviation. Whatman paper and newspaper have the lowest average percentage and lowest standard deviation, with no lipids found in either biomass.

Kjeldahl calculations represent percentage of protein. The results show that rhubarb seems to contain the highest percentage of protein with the standard deviation of 0,43. However, Whatman paper and newspaper have the lowest percentage of protein (0%), with the standard deviation of 0, for both hydrolysates.

The calculation of carbohydrates was based on the results from the proximate analysis, that are also shown in table 4. These calculations show that Whatman paper and newspaper have the highest carbohydrate content, around 99%. However, rhubarb only shows 66.79% of carbohydrates.

4.2 Effect of liquid-gas phase ratio on 1,2-PD production

To investigate the impact of liquid-gas phase ratio on the production of 1,2-PD from sugars by *Thermoanaerobacterium thermosaccharolyticium* strain HG-8, the strain was cultivated at three L-G ratios (0.09, 1, and 5,62) as shown in Figure 4.

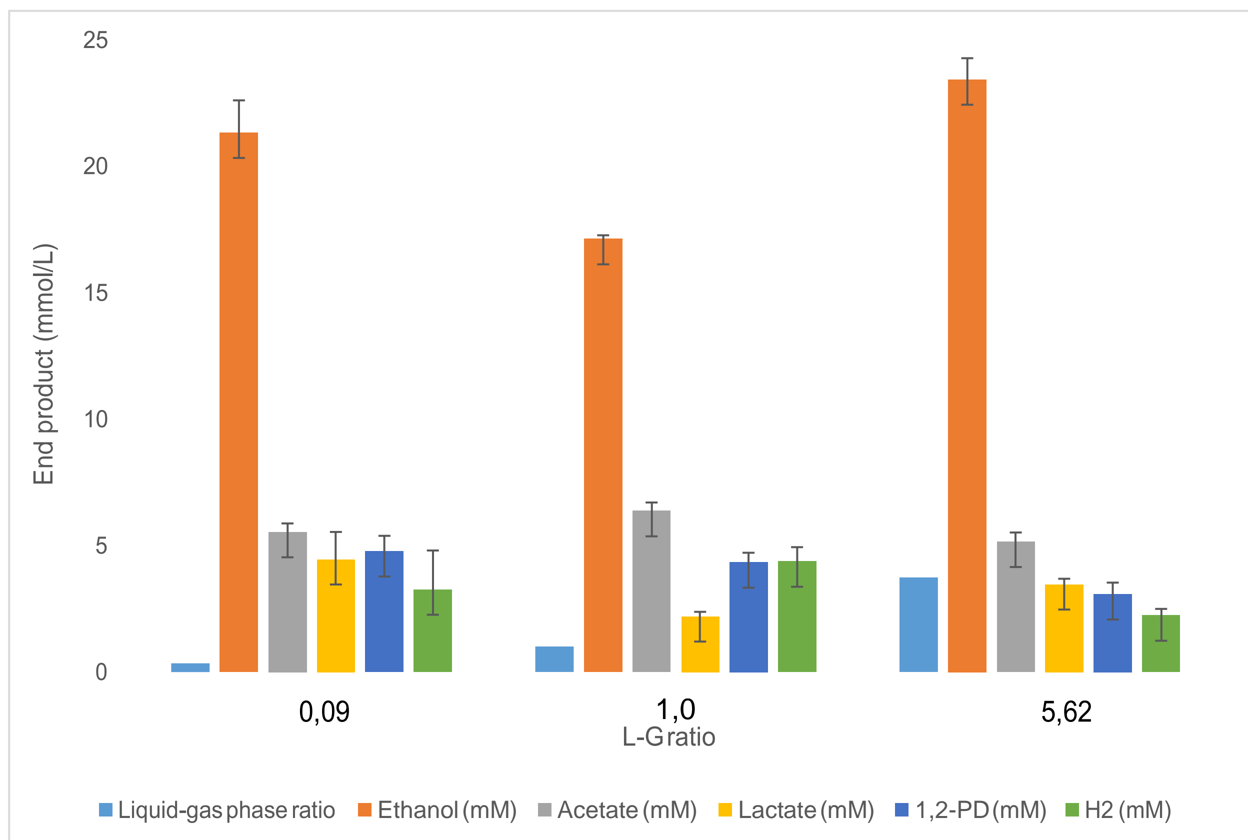

Figure 4 - HG-8 liquid gas phase ratio on 1,2- PD done with three L-G ratio on sugars with standard deviation shown as error bars.

These three ratios had the highest concentration of ethanol in comparison to the other end products available for these L-G ratios. The concentration of ethanol was lowest at 17,13 mM and highest 23,45 mM. Acetate was relatively stable for all the L-G ratios used, with concentrations between 5,51 and 6,37 mM. There were no dramatic changes between any of the ratios, but lactate went from 4,46 mM to 2,2 mM and up again to 3,47 mM. However, 1,2-PD decreased between those ratios, starting from 4,46 mM at the lowest ration and dropping to was 3.08 mM for the highest ratio. H_2 started with 3.27 mmol/L, increased to 4,37 mmol/L and then dropped to 2,24 mmol/L as the ratio increased.

4.2 1,2-PD production from lignocellulosic and algal biomass

End product formation from the fermentation of glucose (20 mM), whatman paper, timothy grass and potato leaf hydrolysates *T. thermosaccarolyticum HG-8* are presented in figure 5.

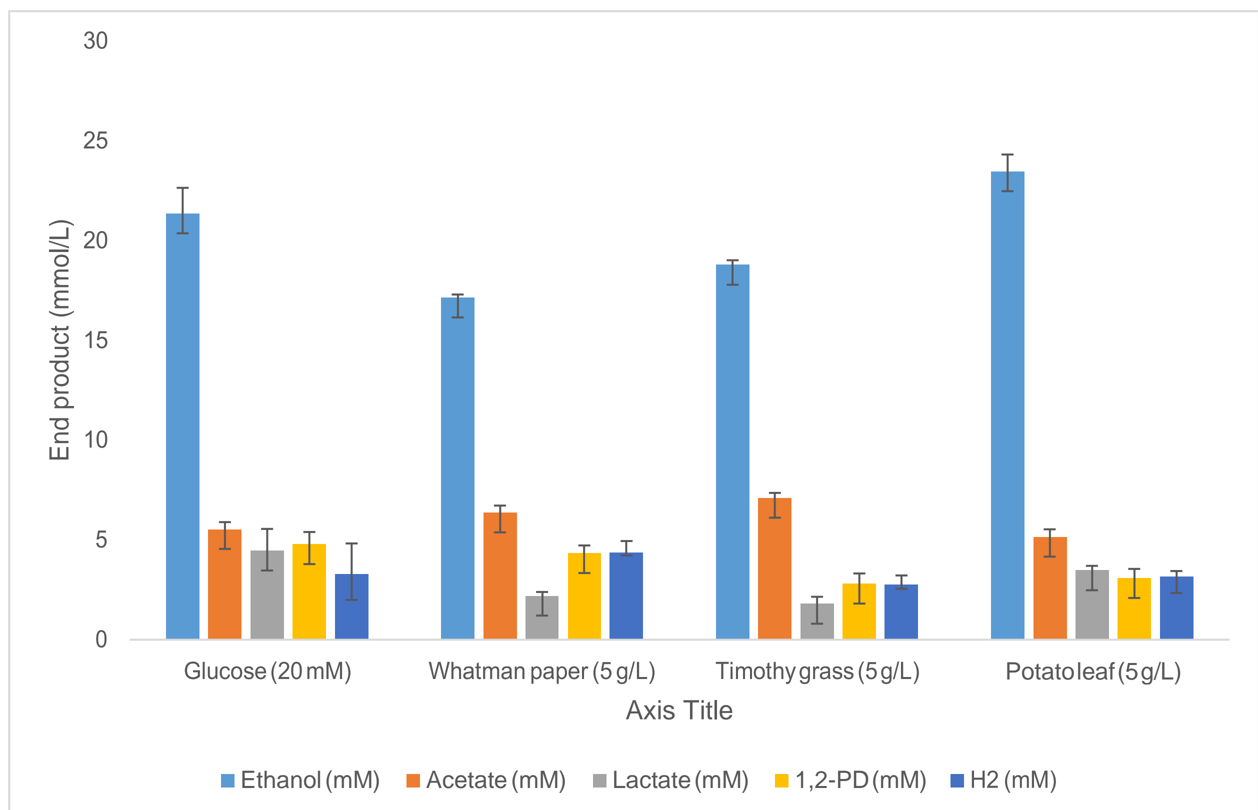

Figure 5 -1,2-Propanediol production by HG-8 on biomass HLs

T. thermosaccarolyticum HG-8 strain formed ethanol, acetate, lactate, 1,2-PD, and H_2 from glucose, whatman paper, timothy grass and potato leaf hydrolysates. All of these hydrolysates produced the highest average yield of ethanol compared to other end products. The other hydrolysates that were made for this experiment, Rhubarb leaf, lupine and newspaper did not show any end product formation above controls. Therefore, the data from these hydrolysates are not shown. The highest concentration of ethanol was formed with potato leaf (23,45 mM) but the lowest concentration was with whatman paper (17,13 mM). The lowest average yield was however lactate, were the highest concentration of lactate was formed with glucose (4,46 mM) but the lowest concentration was with timothy grass (1,8 mM).

The average production yield of 1,2-PD was 4,25 mM, were the highest concentration of 1,2-PD was glucose (4,78 mM) but the lowest concentration was timothy grass (2,81 mM).

4.3 Kinetic experiment

The results for the kinetic experiment shows a connection between time and yield for the production of acetate, lactate, 1,2-PD and H_2. Results for kinetic experiment for glucose can be seen in Figure 6.

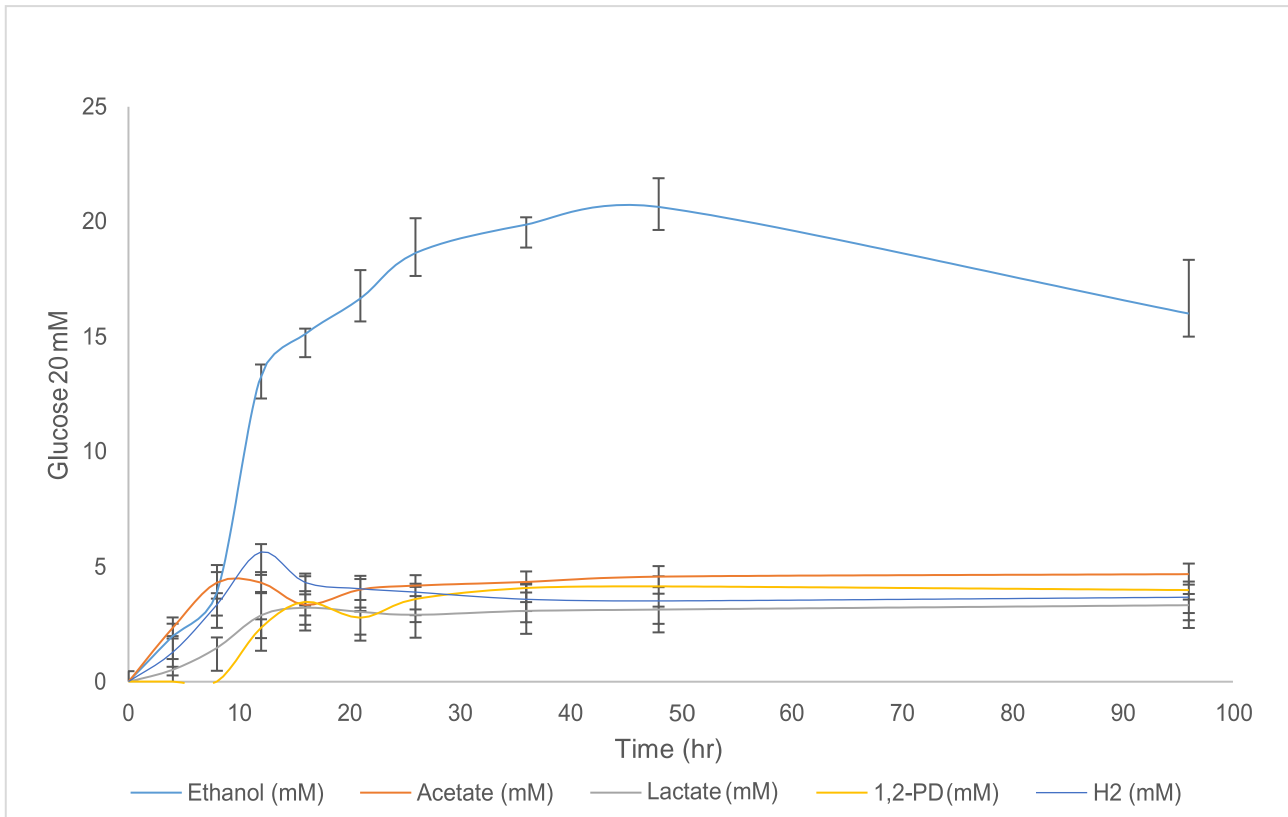

Figure 6 - Results for fermentation of Glucose (20 mM) with Thermoanaerobacterium thermosaccharolytiucm HG-8

The end products reach their maximum potential at different hours. Ethanol for example reaches its maximum potential after 48 hours and is at 20,64 mM. However, after the next 48 hours, it drops down to 16,00 mM and therefore loses 22.48% of its yields. 1,2-PD is at its maximum potential after 48 hours, then it is at 4,14 mM. During the next 48 hours, that are left, it drops down to 3,98 mM and therefore loses 3,86% of its yield.

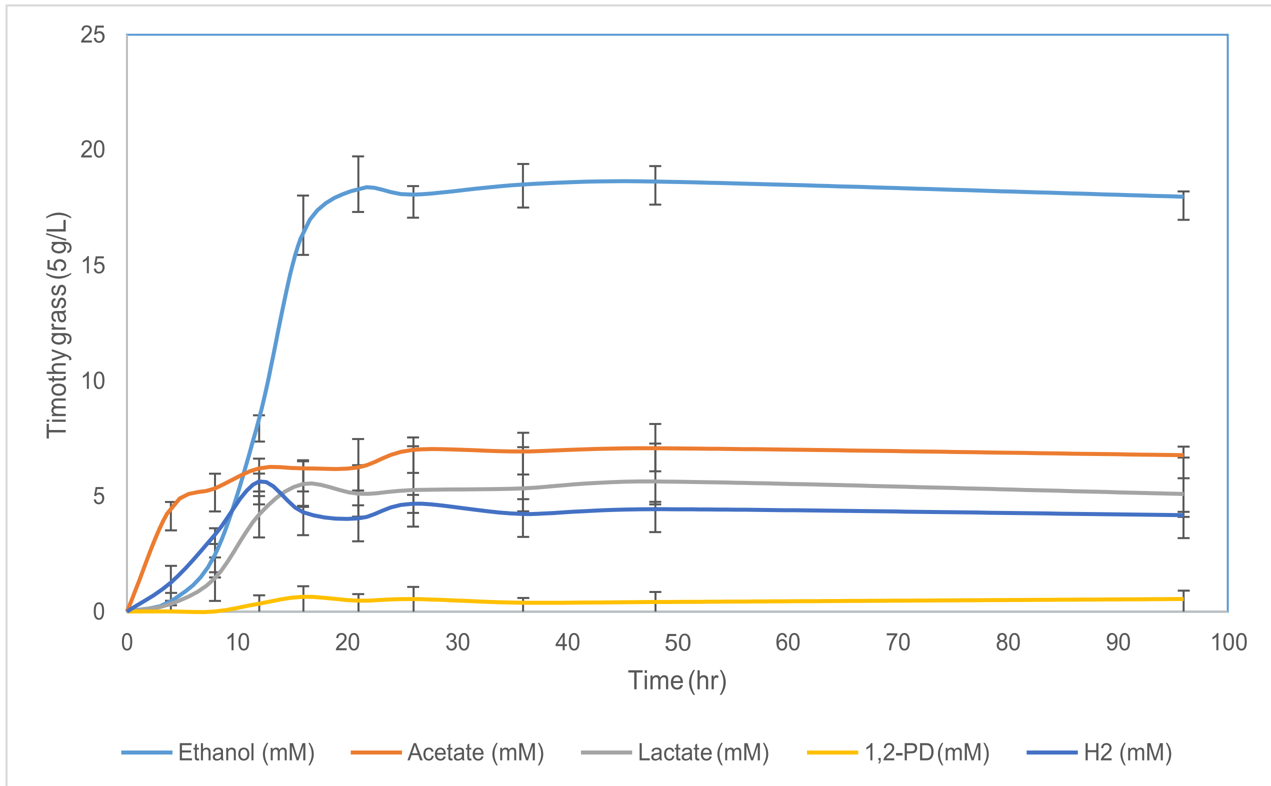

Figure 7 - Results for fermentation of Timothy grass (5 g/L) hydrolysates with Thermoanaerobacterium thermosaccharolytiucm HG-8

The end products reach their maximum potential at different hours. Ethanol for example reaches its maximum potential after 48 hours and is at 18,64 mM. However, after the next 48 hours, it drops down to 17,98 mM and therefore loses 3,54% of its yields. 1,2-PD is at its maximum potential after 16 hours, then it is at 0,64 mM. During the next 80 hours, that are left, it drops down and rises again, but in the end it has dropped down to 0,54 mM or lost 15,6% of its yield.

5 Discussion

5.1 Proximate analysis

The water content in these six different biomasses indicated that dry weight biomass was used. Protein content was rather high for rhubarb, potato leaf and lupine. These results are not that unexpected, except for the rhubarb, that is known for high concentration of oxalic acid, therefore high content of minerals. When rhubarb is eaten it is rather sweet and that should say something about its carbohydrates concentration. However, the results of the carbohydrates for rhubarb show the lowest percentage of all of the hydrolysates.

Whatman paper and newspaper show low or no concentration in ash, Soxhlet and kjeldahl but have high concentration of carbohydrates. This could be due to the fact that these are both papers that probably have been chemically treated. Therefore, all of the minerals, lipids and proteins have been removed according to this experiment.

Timothy grass had a high concentration of minerals or 13,32%. This result is unexpected, although rhubarb and potato leaf also seem to have a high concentration of minerals according to this experiment. Like said earlier, rhubarb is known for high concentration of salt, but potato leaf should be in the same category as the timothy grass. These types of biomasses are not known for high content of minerals, but soils are known for high concentration of minerals. A cross contamination should not be possible, due to the fact that all of the biomasses were washed thoroughly before drying. Therefore, the causes of high concentration mains unknown.

5.2 Effect of Liquid-gas phase ratio on 1,2-PD production

The strain *T. thermosaccharolyticum* HG-8 does not seem to be sensitive to the change of hydrogen accumulation. The results from the different ratio do not shown much changes for any of the end products, including 1,2-PD.

L-G phase ratio has been demonstrated to be of critical importance with strain *T. thermosaccharolyticum* AK68. Were the high L-G phase ratio lead to the formation of high yields of ethanol and low yield of acetate. However, low L-G phase ratio lead to the formation of acetate and hydrogen as the dominant products. This underlines the importance of L-G phase ratio in terms of inhibition and influencing end product formation patterns (Vipotnik, Jessen, Scully, & Orlygsson, 2016).

5.3 1,2, PD production from lignocellulosic and algal biomass

T. thermosccharolyticum HG-8 is a good choice for 1,2-PD from lignocellulosic and algal biomass due to the fact that it can ferment a variety of sugars, that can be found in biomass and the fact that the strain is thermophilic is also a desirable property (Altaras et al., 2001). The variety of sugars that this strain can produce 1,2-PD from is shown in table 1.

The results shown in Figure 5, show that not all of the biomasses had the potential to produce some of the end products. These biomasses that had poor results were rhubarb, lupine and new paper. Their results show that acetate was rather high and could have had impact on the results of the end product. The most likely reason for high yields of acetate is that the yeast extract in the medium got fermented in the meantime. Research has shown that acetate is known for its inhibition of other end products, if its yield is high (Hu, Zhao, Zhao, Wu, & Zhao, 2009).

Although, acetate seems to be the answer for the inhibition of other end products, there is another explanation, at least for the newspaper. Newspaper has already been treated with chemical or physical treatment, therefore it does not require the extensive pretreatment that are developed for lignocellulosic materials, like the other biomasses. Other difference between newspaper and raw lignocellulosic materials, is the fact that newspaper includes ink and fillers, that are added in the paper-making process. These added chemicals could potentially interfere with enzymatic hydrolysis of newspaper. Before being able to use newspaper as a carbohydrate source, ink and other chemicals need to be removed. Therefore, a pretreatment process was developed that has shown to be extremely effective at removing ink from newspaper (Kim & Moon, 2003).

Rhubarb has a high concentration of oxalic acid. Oxalic acid is a corrosive acid that is present in many plants. Oxalic acid is also known to form an insoluble salt with calcium (Oke, n.d.). Rhubarb is known for high concentration of calcium, therefore, the high concentration of insoluble salt measured in rhubarb can be explained from that fact. In this experiment rhubarb showed 11,93% mineral concentration. This mineral concentration can be due to oxalic acid, that could possibly be an inhibiting factor.

In this experiment the highest end product was ethanol for all of the hydrolysates, that have been shown in figure 4. These hydrolysates are whatman paper, timothy grass and potato leaf. This figure also includes the sugar glucose. Glucose was included in this experiment due

to the fact that it is known to produce 1,2-PD with *T. thermosaccarolyticum* HG-8 strain. Glucose was used as criteria for the production of end products.

These hydrolysates have the average production yield of 1,2-PD at 3.41 mM, while glucose produced 4,78 mM. These results show that none of these hydrolysates have as good 1,2-PD producers as glucose. Although, this can be confusing, due to the fact that these hydrolysates probably contain more than one sugar. Each sugar then has lower concentration than the criteria, that is fermented to end product.

The total percentage of carbohydrates is shown in table 7. Total concentration of carbohydrates in whatman paper is 99,95%, 82,40% for timothy grass and 70,52% for potato leaf. Aside from glucose, Whatman paper had the best yield of 1,2-PD in the end product. Therefore, percentage of carbohydrate could be connected to the final yield of 1,2-PD. Although, in this experiment, Timothy grass had the lowest yield of 1,2-PD but did not have the lowest concentration of carbohydrates. That indicates that the concentration of which sugars are present in the biomass, matters to the production of the end product. For example, Potato leaf have lower percentage of carbohydrates, than Timothy grass, but produces higher yields of 1,2-PD. That could indicate that Potato leaf has higher concentration of sugars which can be converted to 1,2-PD. These results could indicate that some biomasses can be a good substrate for producing 1,2-PD. Proper experiment of de-oxy concentration in biomasses is needed before being able to use lignocellulosic biomasses as a substrate for production of 1,2-PD due to the fact that different sugars produce multiple yields with variety of strains.

Original work with *T. thermosccharolyticum* HG-8 shows yields of 9,0 g/L (69,705 mmol/L) of 1,2-PD on glucose. However, glucose in this experiment only yielded 4,48 mmol/L of 1,2-PD (Cameron, Altaras, Hoffman, & Shaw, 1998). The difference between these two experiments is extremely high.

The production of 1,2-PD is different between strains and de-oxy sugars. To compare HG-8 to *C. sphenoides* produces 72,6 mM with rhamnose and 68,6 mM with fucose, while HG-8 produces 68,705 mM with glucose and 27,1075 mM with galactose. *S. cerevisiae* produces 1,8588 mM with glucose and *E. coli* produces 77,45 mM with glucose. Of these strains, E. coli has the highest yields of 1,2-PD. Results for other strains can be seen in table 1.

5.4 Kinetic experiment

The results from the kinetic experiment show that 1,2-PD does not appear until relatively late into the fermentation. That goes for both glucose and Timothy grass. No appearance of 1,2-PD is in the first 12 hours. The first appearance of 1,2-PD for glucose is after 12 hours and it shows yield of 2,34 mM. Glucose reaches maximum yield of 1,2-PD when 48 hours have passed, then the yield has reached 4,14 mM. The first appearance of 1,2-PD for Timothy grass is also after 12 hours, then the yield goes up to 0,34 mM, however, it reaches the maximum after 21 hours and has a yield of 0.64 mM. The difference of maximum yields from glucose and Timothy grass for 1,2-PD is 3,5 mM.

Other end product that are fermented with glucose and timothy grass all have that in common that they have all appeared after 4 hours into the fermentation. H_2 reaches maximum yields after 12 hours for both glucose and timothy grass and the yield is at 5,64 mmol/L. Other end products than H_2 reach their maximum yields later into the fermentation, such as acetate. Acetate reaches it maximum yield at 96 hours for glucose and the yield is at 4,68 mM. For timothy grass, acetate reaches it maximum yield at 48 hours and has the yield of 7,08 mM.

6 Conclusions

The production of 1,2-PD from sugars with *T. thermosaccarolyticum* HG-8 is possible, but there are factors that need to be thought of before deciding to start production of 1,2-PD. These factors include the choice of sugars or renewable lignocellulosic biomass, due to the fact that different types of sugars produce different amount of 1,2-PD and lignocellulosic biomass often have inhibitory compounds included.

The fermentation of renewable biomasses to 1,2-PD by HG-8 provides the basis for important and environmentally friendly process for the production of a biofuel from a renewable source. In this experiment, whatman paper showed the most promise to the production of 1,2-PD, due to high concentration of carbohydrates.

The strain HG-8 does not seem to be sensitive to the change of hydrogen accumulation in the liquid-gas (L-G) phase ratio.

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Appendix

BM medium

Ingredient for 1 liter

Volume	Materials
845 mL	dH_2O
50 mL	1 M PO_4 Buffer (pH 7)
5 mL	Resazurin
2 g	Yeast extract

C_1

Component	for 50 mL	for 100 mL	for 200 mL
Solution G	1 mL	2 mL	4 mL
Solution F	1 mL	2 mL	4 mL
Solution D	12,5 mL	25 mL	50 mL
Distilled water	35,5 mL	71 mL	142 mL

C_2

Component	for 10 mL	for 50 mL	for 100 mL	for 200 mL
Solution E	9 mL	45 mL	90 mL	180 mL
Cysteine HCl	0,5 g	2,5 g	5 g	10 g
Solution H	1 mL	5 mL	10 mL	20 mL